

AD _____

Award Number: DAMD17-02-1-0002

TITLE: Derivation of Double-Targeted Adenovirus Vectors for Gene
Therapy of Prostate Cancer

PRINCIPAL INVESTIGATOR: Victor Krasnykh, Ph.D.

CONTRACTING ORGANIZATION: The University of Alabama at Birmingham
Birmingham, Alabama 35294-0111

REPORT DATE: January 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE January 2004	3. REPORT TYPE AND DATES COVERED Annual (15 Dec 02-14 Dec 03)	
4. TITLE AND SUBTITLE Derivation of Double-Targeted Adenovirus Vectors for Gene Therapy of Prostate Cancer			5. FUNDING NUMBERS DAMD17-02-1-0002	
6. AUTHOR(S) Victor Krasnykh, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The University of Alabama at Birmingham Birmingham, Alabama 35294-0111 E-Mail: vkrasnykh@di.mdacc.tmc.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) The subject of these studies is the development of targeted vector for selective infection and killing prostate cancer cells. The resultant vector is to be administered to prostate cancer patients to find, infect and destroy tumor cells. Targeting of the vector to prostate tumors is to be accomplished via incorporation into its capsid of ligands, which selectively bind to prostate-specific membrane antigen (PSMA). Research accomplishments: (i) a panel of anti-PSMA antibodies has been derived; (ii) a single chain version of one of these mAbs has been incorporated into a phage to serve as a PSMA-binding ligand; (iii) ELISA- and FACS-based procedures have been established to allow for detection of PSMA-bound vectors. Based on the results of Year 1 and 2 studies, a change in the workplan is proposed.				
14. SUBJECT TERMS Prostate cancer, adenovirus, targeting, phase library biopanning, prostate specific membrane antigen, osteocalcin				15. NUMBER OF PAGES 10
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

20040217 034

Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	5
Reportable Outcomes.....	5
Conclusions.....	5
Appendix.....	7

Introduction

The subject of the present studies is the development of adenovirus (Ad)-based vector capable of selectively infecting and killing prostate cancer cells. The intended use of such a vector is for gene therapy of prostate cancer patients, whereby the virus administered to patients would find, infect and destroy tumor cells. Development of this vector is to employ the concepts of conditionally replicative Ad (CRAd) and genetic targeting of Ad to tumor-specific cell surface molecules (transductional targeting). Conditional control over the replication of the vector is to be achieved by using the previously established osteocalcin (OC) promoter. Targeting of the virus to prostate tumor cells will be accomplished via genetic incorporation into its capsid of peptide ligands, which selectively bind to a major marker of prostate tumors, prostate-specific membrane antigen (PSMA). Identification of these targeting peptides by phage library biopanning is the central task of the project. The efficiency of the resultant Ad therapeutic is to be tested by using the virus for the treatment of prostate tumor xenografts established in immunodeficient mice.

Body

The ultimate goal of the proposed studies, the generation of highly specific CRAd for treatment of PCA, was to be achieved via realization of the following specific aims.

Specific Aim 1. To identify PSMA-specific peptide ligands by means of phage display technology.

Specific Aim 2. To derive recombinant Ad5 vectors targeted to PCA cells via genetic incorporation PSMA-specific peptides in the Ad fiber protein.

Specific Aim 3. To generate CRAd derivatives of PSMA-targeted vectors and demonstrate their oncolytic advantages over parental CRAd vector *in vitro*.

The actual progress in the progress is outlined below.

According to the original timeline, during the Years 1 and 2 of the project we were to complete Specific Aims 1 and 2 and also start the work outlined in Specific Aim 3.

However, as it has been reported previously (see our Year 1 progress report), beginning Year 1 the progress in the project has been behind the timeline outlined in the original application. The major reason for that was that, contrary to our expectations, we have not been able to prove that the peptide ligands identified using commercially available phage display libraries and pPSMA-expressing cells indeed bind PSMA protein. At the end of the Year 1 of the project we have shown that the populations of phage eluted from PSMA-expressing cells is enriched for phage species each displaying one of the six different peptide motifs: **ALPQWLL**, **ALPSWLL**, **KLWVIPQ**, **KLWSIPR**, **SAVHLSA**, and **HTVGASS**. At that point we hoped that subsequent studies would confirm the specificity of at least some of these peptides for PSMA, which would eliminate the bottleneck in the project and would thus allow us to complete the entire program within the specified timeline.

However, when the phage species representing the aforementioned peptides were tested for binding to the PSMA-expressing cells in Year 2 studies, none of them demonstrated any binding to these cells above background level. Based on these finding we chose to exclude these motifs for the study.

As the project could not move on without targeting ligands being identified, we have continued our phage biopanning efforts in the Year 2 of the study. This time, instead of relying on the libraries available to us from commercial vendors, we chose to switch to libraries and biopanning

methods developed by others. To this end, we have established two key collaborations with scientists whose expertise in the field of biopanning was significantly better than ours. Specifically, we worked on this problem together with Dr. Renata Pasqualini (University of Texas, MD Anderson Cancer Center, Houston, TX) and Dr. Victor Romanov (State University of New York at Stony Brook, Stony Brook, NY). Unfortunately, each of these collaborations ended up the same way as our own effort in the Year 1 did: while some "consensus" peptide motifs were seen in the enriched library pools after several rounds of biopanning, none of those peptide species showed any significant binding to the cell-anchored PSMA when tested individually. Similarly, negative results were obtained with these phage clones in ELISA utilizing recombinant Fc-PSMA protein.

Key research accomplishments

At this juncture, the key research accomplishments in the project are those listed in the Year 1 progress report.

Reportable outcomes

The results of our studies described herein constituted a part of the poster presentation entitled *Targeting of adenovirus employing a protein bridge approach combined with genetic modification of the viral capsid*, which was presented by Ms. Belousova at the Vector Targeting: Therapeutic Strategies for Gene Therapy meeting at Cold Spring Harbor in March 2003.

Conclusions

The failure with the identification of the PSMA-specific peptides has not allowed us to make progress in the project and use the peptides for making PSMA-targeted CRADs, and thus has become a bottleneck of the entire project. After having spent as much time, labor and money as we have, we believe that continuing this project along the lines of the original workplan is counterproductive.

Despite the fact that the objectives of the original proposal have not been realized, some of the data we have generated lead us to believe that the goal of developing a PSMA-targeted therapeutic vector may still be realized within the original timeframe of the proposal. While working on this project, we have developed a phage vector, which is targeted to PSMA and may serve as a prototype of a therapeutic agent to treat PCA. Specifically, by immunizing mice with recombinant Fc-PSMA protein we generated a panel of hybridomas expressing monoclonal antibodies (mAb), which specifically bind to the extracellular domain of cell-associated PSMA. We then used RNA isolated from one these hybridomas, designated C6C, to derive a single chain version of this mAb (scFv) and displayed it on the surface of a bacteriophage vector for subsequent characterization. The ability of this phage clone to bind to PSMA was confirmed in three assays: ELISA on recombinant Fc-PSMA, ELISA on fixed PSMA-expressing cells, FACS on PSMA cells. Also, the sequence of the PSMA-specific scFv cDNA has been determined. These results have been included into our Year 1 progress report to the DOD PCRP.

Based on these findings, we would like to propose the changes in the project, which are outlined in the Appendix.

List of personnel receiving pay from the award

Victor Krasnykh

Natalya Belousova

Note: due to the relocation of the PI's laboratory from the University of Alabama at Birmingham to University of Texas MD Anderson Cancer Center, the work on this project in Year 2 was limited to a period of January-August 2003.

Appendix.

Proposed new workplan.

HYPOTHESIS/RATIONALE/PURPOSE

The recent studies demonstrated that filamentous phage vectors are capable of delivering the transgenes to and expressing them in the eukaryotic cells (1-4, 7). Additionally, PSMA has been shown to possess internalization function and being capable of internalizing PSMA-specific antibodies (5). Based on these findings, **we hypothesize that the PSMA-specific phage vector designed in the Year 1 of this project can be employed for targeted gene delivery to PSMA-expressing PCA cells and thus serve as prototype of a therapeutic vector.**

OBJECTIVES

The aforementioned hypothesis will be tested via realization of three specific aims:

1. We will modify the genome of the previously designed PSMA-targeted phage to contain a gene cassette capable of directing the expression of the fusion protein comprising a light reporter (green fluorescent protein, GFP) and a therapeutic gene (herpes simplex virus thymidine kinase, HSV TK) in mammalian cells.
2. We will use this phage to demonstrate the capacity of the phage to transduce PSMA-positive human cells by virtue of binding to PSMA and exploiting its natural capacity for internalization. This will be done by using the phage-induced GFP fluorescence in the transduced cells.
3. We will demonstrate the therapeutic utility of this vector by selectively eradicating PSMA-expressing cells *in vitro*. This will be accomplished by exploiting the HSV TK expressed by the phage in combination with the pro-drug (ganciclovir) treatment

METHODS

Specific Aim 1.

Rationale: The genome of the PSMA-targeted phage, which has been generated in the project does not have any gene cargo incorporated, therefore the gene transfer by this phage is difficult to track *in vitro* and especially *in vivo*. Furthermore, as designed, it does not have any therapeutic utility due to the lack of any therapeutic gene payload. These deficiencies of the vector will be overcome by the development of its derivative, whose genome will be modified to contain a gene cassette which will provide the expression of the fusion protein comprising a light reporter (green fluorescent protein, GFP) and a therapeutic gene (herpes simplex virus thymidine kinase, HSV TK) in mammalian cells. While the GFP component of the fusion protein will serve as a light reporter and will thus allow us to monitor the gene delivery and expression by the phage, the HSV TK moiety will serve the purpose of a therapeutic modality by providing the pro-drug(ganciclovir)-activation function.

Experimental plan: The expression cassette encoding HSV TK/GFP fusion will be PCR-amplified using the previously designed plasmid vector SFG-*nesTKGFP* (6) as a template and cloned into the pSEX81.C6C phagemid, which encodes the anti-PSMA scFv fused to pIII protein of the phage. The sequence of the cassette within the modified phagemid will be confirmed by DNA sequencing. Functional validation of the expression cassette incorporated into the

phagemid will be done by transfection of 293 and LNCaP cells and monitoring GFP expression with fluorescent microscope.

The phage particles displaying the C6C scFv and expressing the HSV TK/GFP fusion will be generated by infecting the phagemid-containing bacteria with the helper phage as described in our Year 1 progress report. The phage particles will be purified and the titer of the phage will be determined with a spectrophotometer.

Specific Aim 2.

Rationale: While specific binding of the C6C scFv-containing phage to PSMA has been demonstrated early in the project, the ability of this phage to deliver the transgenes to and express them in human cells has not been demonstrated yet. As this capacity is the key feature of a gene therapy vector, it has to be clearly demonstrated to ensure that the phage is indeed suitable for gene transfer.

Experimental plan:

Human embryonic kidney cells 293 (PSMA-negative) and their PSMA-expressing derivative, 293-PSMA, will be used as targets for C6C-targeted phage. The cells grown in monolayer will be treated with the phage at various multiplicities of infection (MOI) ranging from 10 to 10^4 phage particles per cell. The efficacy of the gene transfer and the temporal profile of gene expression will be assessed by viewing the cultures under fluorescent microscope. Additionally, the percentage of the transduced cells and the magnitude of the gene expression will be addressed by FACS analysis of the transduced cells. These experiments will determine the minimal MOI of infection resulting in 100%-efficient transduction of PSMA-positive cells and will give us an estimate of the level of non-specific transduction (data obtained with 293 cells).

These experiments will then be repeated using LNCaP human prostate cancer cells as target for the phage. As PSMA-negative version of this cell line is not available, to confirm the PSMA-specificity of the phage-mediated gene transfer to LNCaP, we will employ parental C6C mAb and soluble Fc-PSMA protein as specific competitors of phage binding to cell-associated PSMA. In the aggregate, these experiments will show the capacity of the phage to transduce PSMA-positive cells via PSMA-mediated entry pathway and will determine the MOI necessary to achieve 100%-efficient cell transduction.

Specific Aim 3.

Rationale: Having confirmed that the PSMA-targeted phage is capable of gene transfer to PSMA-expressing cells we will need to show that this gene transfer capacity of the vector may be translated into a therapeutic feature by engaging the pro-drug converting activity of phage-expressed HSV TK. Additionally, it will be important to show that the expression of HSV TK mediated by the targeted phage combined with the ganciclovir treatment results in the so-called bystander effect and leads to eradication of those tumor cells which have not been infected by the phage.

Experimental plan: First, LNCaP cells will be infected with the targeted phage at MOI resulting in 100% transduction and the cells will be treated by ganciclovir. The efficacy of the cell killing will be assessed by fluorescent microscopy and XTT cell viability assays.

Subsequent to that, we will show the bystander effect-mediated killing of LNCaP cells in a classical mixing experiment whereby LNCaP cells will be transduced with the targeted phage at 100% efficiency and, upon removal of the phage, will be mixed at different ratios with

untransduced LNCaP cells. Again, the cell killing will be monitored by fluorescent microscopy and XTT cell viability assays.

REFERENCES

1. Ivanenkov, V. V., and A. G. Menon. 2000. Peptide-mediated transcytosis of phage display vectors in MDCK cells. *Biochem Biophys Res Commun* 276:251-7.
2. Larocca, D., and A. Baird. 2001. Receptor-mediated gene transfer by phage-display vectors: applications in functional genomics and gene therapy. *Drug Discov Today* 6:793-801.
3. Larocca, D., M. A. Burg, K. Jensen-Pergakes, E. P. Ravey, A. M. Gonzalez, and A. Baird. 2002. Evolving phage vectors for cell targeted gene delivery. *Curr Pharm Biotechnol* 3:45-57.
4. Larocca, D., K. Jensen-Pergakes, M. A. Burg, and A. Baird. 2001. Receptor-targeted gene delivery using multivalent phagemid particles. *Mol Ther* 3:476-84.
5. Liu, H., A. K. Rajasekaran, P. Moy, Y. Xia, S. Kim, V. Navarro, R. Rahmati, and N. H. Bander. 1998. Constitutive and antibody-induced internalization of prostate-specific membrane antigen. *Cancer Res* 58:4055-60.
6. Ponomarev, V., M. Doubrovin, I. Serganova, T. Beresten, J. Vider, A. Shavrin, L. Ageyeva, J. Balatoni, R. Blasberg, and J. G. Tjuvajev. 2003. Cytoplasmically retargeted HSV1-tk/GFP reporter gene mutants for optimization of noninvasive molecular-genetic imaging. *Neoplasia* 5:245-54.
7. Poul, M. A., and J. D. Marks. 1999. Targeted gene delivery to mammalian cells by filamentous bacteriophage. *J Mol Biol* 288:203-11.

Administrative note: As the strategy of the proposed work will change and become substantially different from the originally proposed one, we suggest that the title of the project is also changed. We would like to suggest the "*Derivation of targeted phage vectors for gene therapy of prostate cancer*" as the new official title for this proposal.

Statement of Work

Derivation of targeted phage vectors for gene therapy of prostate cancer

Task 1. To modify the genome of the previously designed PSMA-targeted phage to contain a gene cassette capable of directing the expression of the fusion protein comprising a light reporter (green fluorescent protein, GFP) and a therapeutic gene (herpes simplex virus thymidine kinase, HSV TK) in mammalian cells (Months 1-2).

Task 2. To employ the PSMA-targeted phage to demonstrate its capacity to transduce PSMA-positive human cells by virtue of binding to PSMA and exploiting its natural capacity for internalization (Months 3-6)

Task 3. To demonstrate the therapeutic utility of this vector by selectively eradicating PSMA-expressing cells *in vitro* (Months 7-12)
